Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids

(macrophage/atherosclerosis/free radical/metal ion/phospholipase)

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ABSTRACT   Low density lipoprotein (LDL) incubated with cultured endothelial cells from rabbit aorta or human umbilical vein is altered in several ways (EC-modified): (*i*) It is degraded by macrophages much faster than LDL similarly incubated in the absence of cells or incubated with fibroblasts. (*ii*) Its electrophoretic mobility is increased. (*iii*) Its density is increased. We report here that antioxidants completely prevent these changes. We also report that these changes do not take place if transition metals in the medium are chelated with EDTA. During EC-modification as much as 40% of the LDL phosphatidylcholine is degraded to lysophosphatidylcholine by a phospholipase A_2-like activity. When incubation conditions in the absence of cells were selected to favor oxidation—for example, by extending the time of incubation of LDL at low concentrations, or by increasing the Cu^{2+} concentration— LDL underwent changes very similar to those occurring in the presence of cells, including degradation of phosphatidylcholine. Hence, some phospholipase activity appears to be associated with the isolated LDL used in these studies. The results suggest a complex process in which endothelial cells modify LDL by mechanisms involving generation of free radicals and action of phospholipase (s).

The lipid-laden foam cells in atherosclerotic lesions are derived largely or in part from monocyte/macrophages (1, 2). These cells have only low levels of the classical low density lipoprotein (LDL) receptor and take up native LDL *in vitro* at relatively low rates, insufficient to cause lipid accumulation to the extent found *in vivo* (3). It has been suggested that this paradox may be explained if the LDL particle is in some way altered *in vivo* to a form taken up more readily than native LDL. Certain chemically modified forms of LDL, including acetylated LDL, are indeed taken up much more rapidly than native LDL by macrophages, and this uptake involves a different receptor, designated the acetyl-LDL receptor (3–5). Incubation of LDL with cultured endothelial cells generates a modified form (or forms) of LDL (endothelial cell-modified LDL; EC-modified LDL) that is taken up 3- to 10-fold more rapidly by macrophages and at least in part by way of the acetyl-LDL receptor (6–8). The modification in biological properties is accompanied by a marked increase in electrophoretic mobility and hydrated density but the mechanisms involved are still poorly understood.

LDL is highly sensitive to metal-catalyzed oxidation (9–11) and oxidized LDL has been shown to be toxic to some cultured cells (12, 13). Endothelial cells in culture have been shown to be capable of oxidizing LDL (14). Since the modification of LDL by endothelial cells involves long incubation under aerobic conditions, we examined the possible role of

oxidative changes in the process. In the present paper, we report that generation of EC-modified LDL is associated with lipid peroxidation and with extensive hydrolysis of LDL phosphatidylcholine (PtdCho) to lysophosphatidylcholine (lyso-PtdCho).

MATERIALS AND METHODS

Materials. Carrier-free $Na^{125}I$ and [1-^{14}C]linoleic acid were purchased from Amersham. Ham's F-10 medium was obtained from Irvine Scientific; α minimal essential medium (α-ME medium) and Dulbecco's modified Eagle's (DME) medium were from GIBCO; and media 199, RPMI 1640, and NCTC 109 were from M. A. Bioproducts (Walkersville, MD). Fetal bovine serum was from HyClone (Logan, UT). Female Swiss Webster mice were supplied by Simonsen Laboratories (Gilroy, CA). Butylated hydroxytoluene was obtained from J. T. Baker Chemical (Phillipsburg, NJ); D-α-tocopherol (vitamin E) was from United States Biochemical (Cleveland, OH); and phospholipase A_2 (from *Ophiophagus hannah* venom) was from Miami Serpentarium (Miami, FL).

Lipoproteins. LDL (ρ = 1.019–1.063) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) and radioiodinated with $Na^{125}I$ using a modification of the ICl method of McFarlane (15); then, the product was dialyzed against phosphate-buffered saline/0.01% EDTA. Protein was determined by the Lowry method (16), using bovine serum albumin as a standard.

Cells. The rabbit aortic endothelial cells used were from a line established and characterized by Buonassisi and coworkers (17). These cells were grown in Ham's F-10 medium/15% fetal bovine serum containing epidermal growth factor at 10 ng/ml in 60-mm plastic culture dishes and were used for experiments at confluence. Resident peritoneal macrophages were isolated from female Swiss Webster mice by lavage with Dulbecco's phosphate-buffered saline. Peritoneal cells were suspended in α-ME medium, with gentamicin sulfate (50 μg/ml), amphotericin B (2.5 μg/ml), and 10% fetal bovine serum and plated at 2×10^6 cells per dish. Nonadherent cells were removed 1 hr after plating, and the adherent macrophages were used in experiments the following day.

Modification of LDL. Endothelial cells were washed three times with serum-free medium and then incubated with 2 ml of serum-free medium containing 100 μg of LDL protein/ml. At the end of the incubation at 37°C (usually 24 hr), the medium was harvested and any detached cells were removed by

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Abbreviations: LDL, low density lipoprotein; EC, endothelial cell; PtdCho, phosphatidylcholine; lyso-PtdCho, lysophosphatidylcholine.
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low-speed centrifugation. The LDL harvested from these incubations is referred to as *EC-modified LDL* although in some of the experiments described below, using inhibitors or different conditions of incubation, the chemical and biological modifications were almost completely prevented. Incubations of LDL were carried out in parallel using dishes without cells (*control LDL*).

Degradation Studies. EC-modified or control ^{125}I-labeled LDL was diluted to 10 μg/ml and then 1-ml portions were added to dishes of cultured macrophages or cell-free dishes. After 5 hr of incubation, the medium was removed and assayed for trichloroacetic acid-soluble noniodide radioactivity as described (6). The macrophages were dissolved in 200 mM NaOH and assayed for protein by the Lowry method.

Lipid Peroxidation Assay. The extent of lipid peroxidation was estimated as thiobarbituric acid-reactive material (18). LDL (25 μg protein) was mixed with 1.5 ml of 0.67% thiobarbituric acid and 1.5 ml of 20% Cl₃CCOOH. After heating at 100°C for 30 min, the reaction product was assayed fluorometrically using a Farrand optical spectrofluorometer with excitation at 515 nm and emission at 553 nm. Freshly diluted tetramethoxypropane, which yields malondialdehyde, was used as a standard and results are expressed as nmol of "malondialdehyde equivalents." The sensitivity of this assay was 0.1 nmol of malondialdehyde equivalents.

Phospholipid Analysis. Lipids were extracted from LDL (100–200 μg of protein) with chloroform/methanol (19), and the solvents were evaporated under nitrogen. The phospholipids were separated by TLC on silica gel G with chloroform/methanol/water, 65:35:8 (vol/vol/vol) and visualized with iodine vapor. PtdCho and lyso-PtdCho bands were scraped from the plates and assayed for phosphorus content (20) and radioactivity.

2-[1-^{14}C]Linoleoyl-PtdCho was prepared by enzymatic acylation of 1-acyl-*sn*-glycerophosphocholine (21) obtained from egg yolk PtdCho by degradation with phospholipase A₂ according to Long and Penny (22). The product, more than 95% pure as judged by TLC and radioactivity scanning, had a specific activity of 3100 cpm/nmol.

Labeled PtdCho (usually 20 nmol) was taken to dryness and then mixed vigorously with 2 ml of F-10 medium. This was added to LDL (200 μg of protein) before incubation with endothelial cells. After incubation, lipids were extracted from the medium in duplicate aliquots and separated by TLC for the determination of phosphate and radioactivity.

RESULTS AND DISCUSSION

Previous studies from this laboratory have shown that cultured endothelial cells can modify LDL in a way that leads to rapid uptake in macrophages and an increase in its electrophoretic mobility and hydrated density (6–8). In the present studies, inclusion of general free-radical scavengers during incubation of LDL with endothelial cells was shown to inhibit modification almost completely. As shown in Fig. 1, LDL incubated with endothelial cells in the presence of butylated hydroxytoluene or vitamin E was degraded no more rapidly by macrophages than LDL from no-cell control dishes. The inclusion of these compounds also prevented the increase in electrophoretic mobility and hydrated density characteristically associated with EC-modification (data not shown). This inhibition was not associated with any evident endothelial cell toxicity; i.e., there was no difference in cell morphology by phase-contrast microscopy, in rate of amino acid incorporation into cell protein, or in rate of LDL degradation nor was there any difference in cell protein recovery at the end of the incubation. The inhibitory effect of the antioxidants was not due to a direct effect resulting from carry-over into the macrophage incubation mixture; direct addition at the concentrations used was shown not to alter macrophage
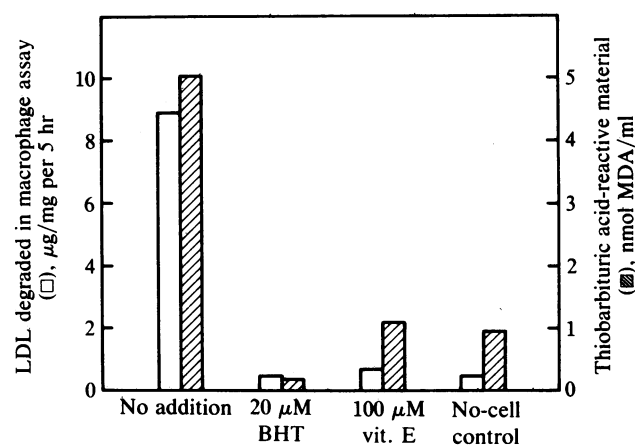

Fig. 1. Inhibition of EC-modification of LDL by antioxidants. ^{125}I-labeled LDL (100 μg of protein/ml) was incubated with cultured rabbit aortic EC or in "no-cell" control dishes for 24 hr in serum-free Ham's F-10 medium with or without antioxidants as indicated (BHT, butylated hydroxytoluene). After this incubation, the rate of degradation of the LDL by cultured mouse resident peritoneal macrophages, as well as the content of thiobarbituric acid-reactive material in the EC-conditioned medium, were determined. Degradation rates are expressed as μg of LDL protein degraded per mg of macrophage cell protein in 5 hr; content of thiobarbituric acid-reactive material is given as nmol malondialdehyde (MDA) equivalents per ml of medium (which contained 100 μg of LDL). Values shown are means of duplicate determinations from a representative experiment.

degradation of modified LDL. Incubation in an argon atmosphere reduced the extent of EC-modification of LDL (data not shown) but did not completely prevent it, possibly because of the difficulty in excluding the last traces of oxygen.

These results indicate that the modification of LDL somehow involved free radical-initiated LDL peroxidation. The extent of lipid peroxidation was directly assessed using the thiobarbituric acid assay and, as shown in Figs. 1 and 2, the generation of thiobarbituric acid-reactive material paralleled the macrophage uptake of LDL. When the LDL was ultracentrifugally reisolated after incubation with the endothelial
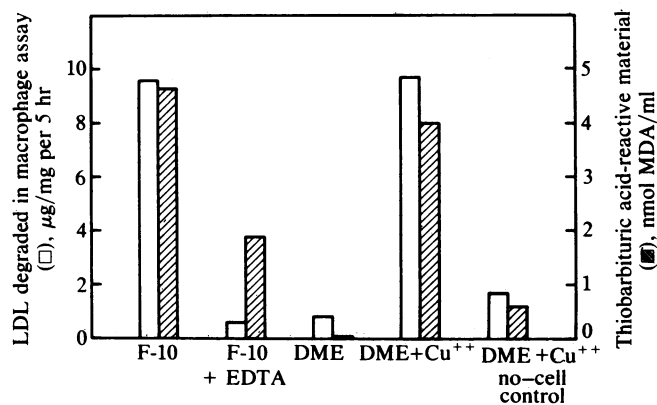

Fig. 2. Dependence of EC modification on transition metals. ^{125}I-labeled LDL (100 μg of protein/ml) was incubated with cultured rabbit aortic EC or in no-cell control dishes for 24 hr. Media were then assayed for rate of LDL degradation by cultured mouse peritoneal macrophages and content of thiobarbituric acid-reactive material as described in the legend to Fig. 1. Incubations were carried out with EC in Ham's F-10 medium without additions, in F-10 medium with 50 μM EDTA, in DME medium (which contains no Cu²⁺), and in DME medium supplemented with 5 μM Cu²⁺. Results are also shown for LDL incubated in no-cell control dishes in DME medium with 5 μM Cu²⁺. Values shown are means of duplicate determinations from a representative experiment. MDA, malondialdehyde equivalents.

cells, it was found that only a small part of the total thiobarbituric acid-reactive material in the medium remained associated with the lipoprotein. Further, more than 75% of the thiobarbituric acid-reactive material could be filtered through a membrane with a molecular weight cut-off of 25,000. These findings might indicate secretion of thiobarbituric acid-reactive substances by the cells or the oxidation and release of lipids from LDL.

In previous studies from this laboratory on the production of EC-modified LDL, incubations were routinely carried out in Ham's F-10 medium (6–8). We now find that the modification does not take place if DME medium is used in place of F-10 even though incubation conditions are in all other respects identical. There is no change in electrophoretic mobility or hydrated density nor in the rate of subsequent degradation when the LDL is transferred to cultured macrophages (Fig. 2). Modification also failed to occur in medium 199, α-ME medium, RPMI 1640, or NCTC 109. This could be due to the presence of inhibitory substances in these media or to the lack of factors such as transition metals that could catalyze peroxidation. Evidence that transition metal ions might be crucial was obtained by demonstrating a complete inhibition of the modification in F-10 medium when 50 μM EDTA was added (Fig. 2). This low concentration of EDTA could complex only a small proportion of the Ca^{2+} and Mg^{2+} but, because of the extremely high association constant of EDTA for Fe^{2+}, Cu^{2+}, and Zn^{2+} and because of their much lower concentrations in the medium, these ions would be almost quantitatively complexed. Further evidence for the importance of metal ions was the finding that supplementation of DME medium with Cu^{2+} allowed modification to occur (Fig. 2). However, the relative importance of different metal ions in EC-modification remains to be established.
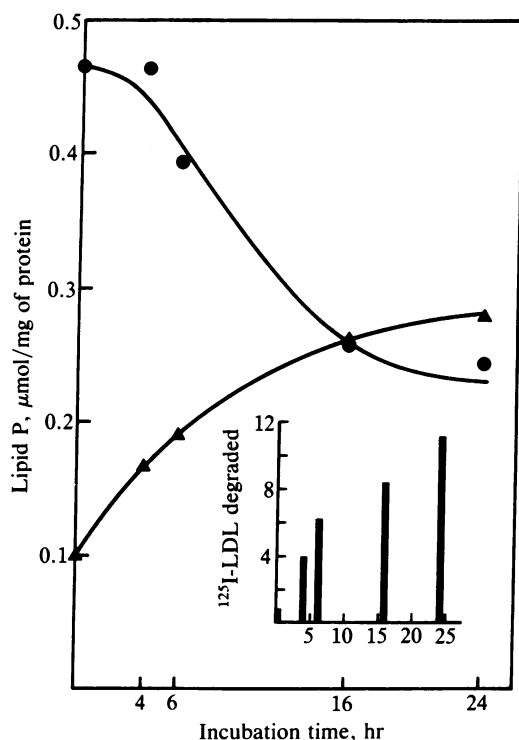
As shown in Fig. 3, the modification of LDL by EC appeared to be accompanied by extensive hydrolysis of the PtdCho in LDL to lyso-PtdCho. The appearance of phospholipid comigrating with lyso-PtdCho could not have been due to oxidized PtdCho as, with the solvent system used, oxidized PtdCho would be expected to move only slightly slower than PtdCho and much faster than lyso-PtdCho (23). Furthermore, the isolated material could be acylated to the parent compound PtdCho, thus establishing its chemical nature as lyso-PtdCho. The phospholipid/protein ratio of EC-modified LDL reisolated by ultracentrifugation was unchanged from that of native LDL, in agreement with previous results from this laboratory (7). Thus, the lyso-PtdCho generated remains associated with LDL under the conditions of the incubation. In some experiments, PtdCho labeled with [1-^{14}C]linoleic acid in the 2-position was added along with unlabeled LDL at the beginning of incubation with endothelial cells and the medium was analyzed at various time intervals (Fig. 4). There was a progressive decrease in PtdCho radioactivity that closely paralleled the decrease in PtdCho mass, indicating that the fate of the labeled PtdCho reflected that of the total PtdCho present in LDL. As much as 40% of the PtdCho in the LDL added had been hydrolyzed by the end of a 24-hr incubation. However, almost no radioactivity was recovered in the lyso-PtdCho, indicating that the labeled 2-position fatty acid and not the unlabeled 1-position fatty acid was cleaved during modification. This specificity suggests that a phospholipase A_2 activity may be involved. Because the fatty acid was labeled at the carbonyl carbon, loss of radioactivity by nonenzymatic scission of the acyl chain at a peroxidized double bond can be excluded. There is no precedent for loss of the entire fatty acyl chain by nonenzymatic oxidative degradation. Most of the released radioactivity remained in the aqueous phase after acidification and extraction with hexane, a procedure that would remove nonoxidized fatty acids. The findings are thus compatible with the interpretation that at least some of the thiobarbituric acid-reactive substances in the medium represent oxidized or per-
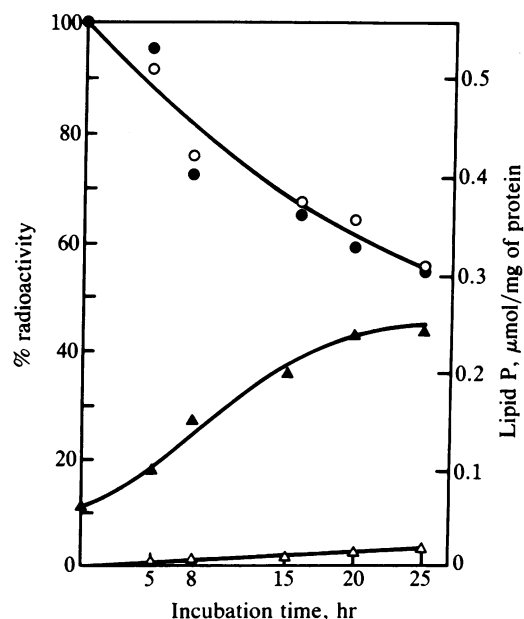

FIG. 3. Hydrolysis of LDL-PtdCho during EC-modification. ^{125}I-labeled LDL (100 μg of protein/ml) was incubated with cultured rabbit aortic EC for various time intervals. EC-modified LDL was isolated from the media by ultracentrifugation after adjusting the density to 1.2 g/ml. Aliquots of modified LDL were taken for assay of PtdCho (●) and lyso-PtdCho (▲) and for macrophage degradation studies. (*Inset*): ^{125}I-labeled LDL degraded is reported as μg/mg of cell protein per 5 hr.

FIG. 4. Hydrolysis of 2-[1-^{14}C]linoleoyl-PtdCho by EC during LDL modification. Rabbit aortic EC were incubated with LDL (100 μg of protein/ml) and 2-[1-^{14}C]linoleoyl-PtdCho (10 nmol/ml) in duplicate dishes. At the time intervals indicated, lipids from the media were extracted, and PtdCho (○, ●) and lyso-PtdCho (△, ▲) were separated by TLC for determination of radioactivity (○, △) and phosphorus content (●, ▲).

oxidized fatty acids cleaved from PtdCho during the course of the incubation. The possibility that a lecithin:cholesterol acyltransferase might be responsible for the hydrolysis of PtdCho was considered since in the absence of an acceptor (cholesterol) this enzyme can act as a hydrolase (24). However, the radioactivity lost from PtdCho was not recovered in the cholesterol ester fraction on TLC. Furthermore, heating LDL at 60°C for 60 min, which is sufficient to inactivate this enzyme, did not prevent EC-modification.

A surprising finding was that the hydrolysis of PtdCho was also inhibited by antioxidants that prevent EC-modification (Table 1). It has been shown that oxidized phospholipids are more susceptible to hydrolysis by phospholipases (25) and this may be the link between the oxidation and the PtdCho hydrolysis noted here. However, this conclusion remains tentative because the antioxidants may have direct effects on the phospholipase activities involved in EC-modification.

In the present studies, incubation periods were limited to 24 hr or less and LDL was added at 100 μg of protein/ml. LDL incubated under identical conditions in the absence of cells shows little or no modification (Fig. 1, control LDL), as previously reported (6, 8). Furthermore, incubation with human fibroblasts or with a line of bovine endothelial cells does not cause modification (7), indicating a measure of cell specificity. However, many or most of the changes observed during incubation with endothelial cells can be duplicated in the absence of cells under appropriate conditions. For example, if low concentrations of LDL are used and the incubation time is extended to 48 hr, the degree of modification becomes comparable with that observed in the presence of cells under standard conditions (Table 2). In view of the exquisite sensitivity of EC-modification to metal ions, the apparent dependency on LDL concentration could reflect in part differences in the amounts of residual EDTA added to the dishes with the LDL preparations. In other experiments, LDL (100 μg of protein/ml) was incubated in the absence of cells for 24 hr in F-10 medium supplemented with 5 μM Cu^{2+}; the degree of modification that resulted was similar to that found in LDL incubated with EC under standard conditions (data not shown). Inhibitory factors in some media may affect the rate of modification by 5 μM Cu^{2+} in the absence of cells. Thus, very little modification was observed after 24 hr of incubation in DME medium with 5 μM Cu^{2+} (Fig. 2) whereas incubation in phosphate-buffered saline with 5 μM Cu^{2+} resulted in very rapid modification (data not shown). Of great interest was the finding that during these incubations in the absence of cells all the changes associated with EC-mod-

Table 1. Inhibition by antioxidants of phospholipid hydrolysis during incubation of LDL with endothelial cells

Incubation conditions	TBA-reactive material, nmol malon-dialdehyde/ml	lyso-PtdCho/PtdCho ratio
EC only	5.1 ± 0.4	0.82 ± 0.12
EC + 20 μM butylated hydroxytoluene	0.3 ± 0.1*	0.22 ± 0.25[†]
EC + 100 μM vitamin E	1.5 ± 1.2[†]	0.15 ± 0.06*
EC + 50 μM EDTA	1.6 ± 0.8[†]	0.14 ± 0.05*
No-cell control	1.2 ± 0.2*	0.19 ± 0.01*

[125]I-labeled LDL (100 μg of protein/ml) was incubated for 24 hr with cultured rabbit aortic EC or in no-cell control dishes in Ham's F-10 medium without additions or with 20 μM butylated hydroxytoluene, 100 μM vitamin E, or 50 μM EDTA. One aliquot of medium was then assayed for content of thiobarbituric acid (TBA)-reactive material, and a second aliquot was taken for phospholipid analysis. Values shown are means ± SD from three separate experiments; significance of differences was assessed with the two-tailed *t* test (*, $P < 0.001$; †, $P < 0.02$).

Table 2. Effect of ^{125}I-LDL concentration on its modification by endothelial cells and in cell-free dishes in prolonged incubations (48 hr)

Incubation conditions	Degradation of ^{125}I-LDL by macrophages*	Electrophoretic mobility, mm
No-cell control, 100 μg of LDL/ml	0.503 ± 0.049	17
EC, 100 μg of LDL/ml	3.74 ± 0.21	29
No-cell control, 20 μg of LDL/ml	4.47 ± 0.20	34
EC, 20 μg of LDL/ml	4.22 ± 0.36	28

[125]I-labeled LDL was incubated for 48 hr in F-10 medium (containing glutamine and gentamicin) in 60-mm dishes containing endothelial cells or in cell-free dishes that had previously been incubated with serum-containing medium. It was then subjected to low-speed centrifugation and the radioactivity in a sample of the supernatant was measured. The concentration of the ^{125}I-labeled LDL was then calculated from its specific activity and aliquots were added to 22-mm dishes of macrophages or cell-free dishes at a concentration of 10 μg of LDL-protein/ml in F-10 medium (containing glutamine and gentamicin). Degradation of the ^{125}I-labeled LDL was determined after 5 hr. Values shown are means ± SD for three dishes of macrophages. The electrophoretic mobility of the control and EC-modified LDL was determined in agarose gels (pH 8.6, 300 V, 30 mA, 2 hr). Whether or not the 60-mm dishes were first incubated with serum-containing medium had no effect on degradation of the ^{125}I-labeled LDL by macrophages.

ification, including degradation of PtdCho, were observed. This finding suggests the presence of a phospholipase activity intrinsic to the LDL as isolated.

At this stage it is not certain whether a phospholipase associated with LDL is playing an essential role in EC-modification or whether the relevant phospholipase activity is associated with the endothelial cells (or both). It is possible that in the course of the incubation of LDL with endothelial cells activation of an intrinsic LDL phospholipase occurs. Alternatively, the interaction of endothelial cells with oxidized LDL may activate cell membrane phospholipases. These cells do have a high level of intrinsic phospholipase activity and can generate prostaglandins at a high rate (26, 27), which could account for generation of some of the thiobarbituric acid-reactive material measured.

Whatever the precise mechanisms involved may be, these findings implicate free radicals and peroxidation (in the medium, in the cells, or both) as a key element in generation of EC-modified LDL. Whether a similar process occurs *in vivo* at a rate significant enough to contribute to atherosclerosis remains to be determined. Preliminary data from our laboratory and from others (28, 29) show that under appropriate conditions cultured smooth muscle cells, stimulated polymorphonuclear cells, and monocyte/macrophages themselves can lead to oxidation of LDL. Such oxidation of LDL might occur in the artery wall, where the levels of antioxidants may be lower than in whole blood. In fact, LDL isolated from the arterial wall (30) and from inflammatory fluids (31) shares some properties of EC-modified LDL but these modified lipoproteins have not yet been completely characterized. If the processes described here do play a role *in vivo*, it would in theory be possible to intervene and alter the atherogenic process by using antioxidants or phospholipase inhibitors or both.

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